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1 2 3 4 5 6 7 8 9 10 11 12 13

A_1 - Trp - Cys - A_2 - A_3 - A_3- X - A_2 - A_3 - A_3 - Cys - A_3 - A_4 (I)

1 2 3 4 5 6 7 8 9 10 11 12 13

Arg-Arg-Trp-Cys-Tyr-Arg-Lys -DLys-Pro- Tyr-Arg-Lys-Cys-Arg-NH_2 (1)

(57) Abstract

A polypeptide represented by formula (I), [in which: A_1 is an amino acid or a peptide having one or at least two basic amino acids selected from lysine, arginine and ornithine, said amino acid or peptide in which amino terminal N-α may be replaced with an acyl group or a substituted thiocarbamoyl group; A_2 is a tyrosine or phenylalanine; A_3 is a lysine or arginine; A_4 is an -OH or an $-NH_2$; and X is a peptide such as D-ornithyl-proline, prolyl-D-ornithine, D-lysyl-proline, prolyl-D-lysine, D-arginyl-proline, prolyl-D-arginine, glycyl-ornithine, ornithyl-glycine, glycyl-lysine, lysyl-glycine, glycyl-arginine and arginyl-glycine, in which the hydrogen atom of the ω-amino group of D-lysine, L-lysine, D-ornithine and L-ornithine may be replaced by an ω-amino acyl group, and said peptide is connected to the amino acid residues at the 6th and the 8th positions via peptide bond per se; Trp is a tryptophan; and Cys is a cysteine], and one example of such polypeptide be represented as formula (1) is presented. The above presented polypeptide may be useful in a pharmaceutical composition as an antimicrobial or antiviral agent, specifically as an anti-HIV agent and as a component of the DNA-transfecting systems for gene therapy.

DESCRIPTION

POLYPEPTIDE AND ANTI-HIV AGENT PREPARED THEREFROM

1. FIELD OF THE INVENTION

This invention relates to a novel polypeptide(s) or a pharmaceutically acceptable salt thereof exhibiting a strong affinity to lipopolysaccharides, particularly endotoxins. The polypeptide may be used in a pharmaceutical composition as an anti-viral agent (e.g. anti-HIV agent).

2. BACKGROUND OF THE INVENTION

Two families of antimicrobial polypeptides have been isolated from horseshoe crabs which exhibit an affinity to endotoxins (see, for example, Shigenaga et al., 1990, J. Biol. Chem. 265:21350-21354; Kawano et al., 1990, J. Biol. Chem. 265:15365-15367; Muta et al., 1990, J. Biochem.108:261-266; Japanese Laid-Open Patent Publication No. 167230/1990; Japanese Laid-Open Patent Publication No. 152987/1990; Japanese Laid-Open Patent Publication No. 53799/1990; Published Searched Application 500194/1990; Miyata et al., 1989, J. Biochem. 106:663-668; Akaji et al., 1989, Chem. Pharm. Bull. 37:2661-2664; Tokunaga and Iwanaga, 1989, Taisha(Metabolism) 26:429-439 ; Shieh et al., 1989, FEBS Lett. 252:121-124; and Nakamura et al., 1988, J. Biol. Chem. 263:16709-16713).

One family, the tachyplesin family has been isolated from the Japanese horseshoe crab Tachypleus. Three tachyplesins, I, II, and III have been identified. A second family, the polyphemusin family has been isolated from the American horseshoe crab, Limulus polyphemus. Two polyphemusins, I and II have been identified; their amino acid sequences are shown in Figure 1.

The polypeptides in both families consist of 17 or 18 amino acid residues and have four conserved regions in common and two disulfide bridges (see Figure 1).

al., 1992, J. Pharmacobio. Dyn., 15, s-90 ; International Laid-Open Publication WO 92/04374 ; Japanese Laid-Open Patent Publication No.163298/1993.
(Hereinafter, among the new polypeptides, the compound T-22 is referred to the best mode compound of the representatives.)
Having examined the structural requirements for exhibiting the activity of the new polypeptide consisting of 16 to 18 amino acids as represented by the T-22 compound, the inventors and coresearchers arrived at some inventive concepts of a minimum essential structure.

In general, when an exogenous peptide of relatively high molecular weight is administered into human body, it is often recognized as an alien substance by the self defence function of the human body. As a result, it is likely to become an antigenic substance. When used for a medical purpose, it is desirable that a peptide-based bioactive substance may be a compound of low molecular weight in order to reduce its likelihood of being recognized as an alien substance. It is also required that the substance may have a high potency.

The T-22 compound was found to be a polypeptide consisting of 18 amino acid residues. The objective of our investigation was to maintain the same level of anti-HIV potency as that of the T-22 compound, while reducing the number of amino acid residues. As a result, we succeeded in reducing the number of residues by 4 (four). As long as the compound has the basic structure, its activity dose not decline even when a specific region is modified.
Moreover, by the said modification, we discovered the novel polypeptide having such essential structure that can provide a broader range of physicochemical characteristics, and also a broader selection in therapeutic methods, i.e. increasing/decreasing of the hydrophilicity and lipophilicity (affinity to lipid); selective accumulation in a specific organ and/or cell; and increasing/decreasing its residence time within the human body; and possible development of formulations.

3. SUMMARY OF THE INVENTION

The present invention relates to a novel polypeptide(s)

(ε-N-ω-aminobutyryl-D-lysine).

The following terms, as used herein, will have the meanings indicated:

HIV = human immunodeficiency virus (all variants)

MOI = multiplicity of infection

SI = selectivity index (ratio of CC_{50} to EC_{50})

4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences of Tachyplesin I, Tachyplesin II, Tachyplesin III, Polyphemusin I, and Polyphemusin II. Conserved amino acids are boxed. The disulfide linkages between Cys-3 or -4 and Cys-16 or -17 and Cys-7 or -8 and Cys-12 or -13 are shown by solid lines.

Figure 2 shows a synthetic scheme for synthesizing polypeptide (1) of the invention.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is accomplished based on the above aspects, and is related to a novel polypeptide represented by the following formula

$$A_1\text{- Trp-Cys - } A_2 \text{ - } A_3 \text{ - } A_3 \text{ - X - } A_2 \text{ - } A_3 \text{ - } A_3 \text{ - Cys - } A_3 \text{ - } A_4 \quad \text{.....(I)}$$

or salt thereof in which

A_1 is a basic amino acid residue, or a peptide residue having one or at least two basic amino acids, selected from the group consisting of lysine, arginine and ornithine, said basic amino acid residue or peptide residue in which N-α hydrogen atom of amino terminal end of said amino acid residue may be replaced with an acyl group or a substituted thiocarbamoyl group, forming N-α acyl substituted basic amino acid residue, N-α acyl substituted peptide residue, N-α substituted thiocarbamoyl group substituted basic amino acid residue or N-α substituted thiocarbamoyl group substituted peptide residue;

A_2 is a tyrosine or phenylalanine residue;

A_3 is a lysine or arginine residue;

TABLE 1

	1	2	3	4	5	6	7	8	9	10	11	12	13
(I)	A_1	- Trp	- Cys	- A_2	- A_3	- A_3	- X	- A_2	- A_3	- A_3	- Cys	- A_3	- A_4
(1)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(2)	Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(3)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Lys-Gly	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(4)	Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Lys-Gly	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(5)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Pro-DLys	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(6)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DOrn-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(7)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Pro-DArg	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(8)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Gly-Lys	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(9)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Arg-Gly	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(10)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- Gly-Arg	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(11)	Lys-Arg	- Trp	- Cys	- Tyr	- Lys	- Arg	- DLys-Pro	- Tyr	- Lys	- Arg	- Cys	- Arg	- NH_2
(12)	Lys-Lys	- Trp	- Cys	- Phe	- Arg	- Lys	- DLys-Pro	- Phe	- Arg	- Lys	- Cys	- Arg	- NH_2
(13)	Arg-Lys	- Trp	- Cys	- Tyr	- Lys	- Arg	- DLys-Pro	- Tyr	- Arg	- Arg	- Cys	- Lys	- NH_2
(14)	Ac -Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(15)	Oct -Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(16)	Laur-Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(17)	Myr -Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(18)	Parm-Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(19)	FTC -Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(20)	PTC -Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
(21)	Nicot-Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
							ϵ-N-Ac						
(22)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
							ϵ-N-But						
(23)	Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
							ϵ-N-But						
(24)	Parm-Arg-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2
							ϵ-N-But						
(25)	Parm-Orn-Arg	- Trp	- Cys	- Tyr	- Arg	- Lys	- DLys-Pro	- Tyr	- Arg	- Lys	- Cys	- Arg	- NH_2

In the n-18 polypeptide, the turning position with possibly beta-turn structure is located at the 9- and 10-positions. And the peptide part of the 3-position to the 8-position and the peptide part of the 11-position to the 16-position face each other.

Like the n-18 polypeptide, the polypeptides of the present invention have an antiparallel β sheet structure due to the existence of intramolecular hydrogen bonding and disulfide linkage (-S-S-) with cysteine residues. While in the polypeptides of the present invention, the turning position with possibly β turn structure is so located in the X at 7th position that the peptide part of the 3-position to the 6-position and the peptide part of the 8-position to the 11-position face each other.

In the present invention, the relationship of the number of amino acid residues at the 1- position of the formula of the present invention is the same as that for the n-18 polypeptide.

It is confirmed that replacement of the hydrogen atom of an α-amino group of the N terminal amino acid residue at the said position by an acyl group or a substituted thiocarbamoyl group is important to exhibit high anti-HIV activity of the novel polypeptide represented by the denoted formula. By selecting different properties of the acyl or the substituted thiocarbamoyl group, it has become possible to give the novel polypeptide of the invention hydrophilicity, affinity for lipids, distinct fluorescence properties, and etc. For example, the fluorescence properties of the fluorescein substituted thiocarbamoyl group in the polypeptides of this invention can be used as a highly sensitive reporter dye for various purposes. See, for example, Brand and Witholt in "Methods in Enzymology," Vol. 11, page 776-856, ed. by Hirs, Academic Press, New York, New York(1967); Brand and Gohlke, 1972, Annu. Rev. Biochem. 41: 843-868; Stryer, 1978, Annu. Rev. Biochem. 47: 819-846.

Moreover, it is very important and useful that the fluorescein substituted thiocarbamoylated polypeptides of this

principle, hydroxyproline may be substituted with proline having the same effect) is necessary factor to form the same planar structure with β- sheet structure. The disulfide side chain linked 3- with 11- positions of cysteine residues and the basic side chains of basic amino acid residues at 5- and 9-positions are on the same side of backbone plane, while the aromatic side chains of aromatic amino acid residues at 4- and 8- positions and the basic side chains of basic amino acid residues at 6- and 10- positions are on the opposite side of backbone plane. Formation of these three-dimensional above mentioned structure is important. Thus the novel polypeptide represented by above denoted formula was invented with such three-dimensional structure, resulting in reduction of 4(four) amino acid residues compared with n-18 polypeptide.

Furthermore, like the n-18 polypeptide(s), the polypeptides of the present invention exhibit very basic characteristics. Due to their basic nature, the polypeptide(s) of the present invention may form a salt by acid addition. For example, the polypeptide forms a salt with an inorganic acid (hydrochloric acid, hydrobromic acid, phosphoric acid, nitric acid, sulfuric acid or the like) or an organic carboxylic acid (acetic acid, halo acetic acid such as trifluoroacetic acid, propionic acid, maleic acid, succinic acid, malic acid, citric acid, tartaric acid, salicylic acid and an acidic sugar (glucuronic acid, galacturonic acid, gluconic acid, ascorbic acid or the like), an acidic polysaccharide (hyaluronic acid, chondroitin sulfate, alginic acid or the like) or an organic sulfonic acid (methanesulfonic acid, p-toluenesulfonic acid or the like) including sulfonic acid sugar ester such as chondroitin sulfates.

The following is a more detailed description of the novel polypeptide of the present invention.

5.1 PREPARATION OF POLYPEPTIDES

The novel polypeptide of the invention can be prepared by methods known in the art, for example, solid-phase synthesis techniques described in "Solid-Phase Peptide Synthesis",

by substituted thiocarbamoyl groups, the N-terminal N-α-substituted thiocarbamoyl polypeptide of the invention can be obtained by reaction with the substituted isothiocyanate compound under slightly alkaline conditions.

In this instance, the carboxyl terminus of the amino acid residue at the 12-position can be either free (A_1 corresponds to -OH) or converted to an acid amide (A_4 corresponds to $-NH_2$). Further, in the obtained polypeptide, the two cysteines at the 3- and 11- positions can form a disulfide linkage (-S-S-) through the mercapto groups.

These disulfide linkage may be formed, for example, by air oxidation, or by the method of Atherton, E., et al., 1985, J. Chem. Soc., Perkin Trans. 1, 2065.

Unless otherwise indicated, the individual amino acid used in the aforementioned solid phase synthesis method is in the L-form, and the basic amino acid coupled with a proline at the 7th position denoted by X is limited to D-form.

Any insoluble resin having an amino group can be used in synthesizing the novel polypeptide of the invention, as long as it can link through its amino groups to the carboxyl group of the N-protected arginine or lysine at the C-terminus or in some cases to the carboxyl group of the spacer linked thereto and thereafter can be eliminated (removed). Examples of such insoluble resins include but are not limited to aminomethyl resins (aminomethylated styrene-divinylbenzene copolymers), benzhydrylamine resins, methylbenzhydrylamine resins and aminomethylphenoxymethyl resins and derivatives thereof. When a benzhydrylamine resin, methylbenzhydrylamine resin, dimethoxybenzhydryl amine (DMBHA) resin or aminomethylphenoxymethyl resin is used, an amide is directly obtained by cleavage, but an aminomethyl resin is preferred in view of yield.

As the spacer having a functional group capable of linking to a carboxyl group or having a carboxyl group, there can, for example, be one that is capable of converting the

according to condensation methods known in the art, such as, for example, a DCC (dicyclohexylcarbodiimide) method, DIPCDI (diisopropylcarbodiimide) method [Tartar, A. et al., 1979, J. Org. Chem. 44: 5000], active ester method, mixed or symmetrical acid anhydride method, carbonyldiimidazole method, DCC-HOBt (1-hydroxybenzotriazole) method [König W. et al., 1970, Chem. Ber., 103: 788, 2024, 2034] or diphenylphosphoryl azide method, but preferably using the DCC method, DCC-HOBt method, DIPCDI-HOBt method or symmetrical acid anhydride method. The condensation reaction may be carried out in an organic solvent such as dichloromethane, dimethylformamide, N-methylpyrrolidone (NMP) or a mixed solvent thereof. A deblocking reagent such as trifluoroacetic acid/dichloromethane, HCl/dioxane, piperidine /dimethylformamide (DMF) or NMP is used to deblock the protecting group for an α-amino group. The degree of the progress of condensation reaction in each step of synthesis is pursued by the method of E. Kaiser et al., 1970, Anal. Biochem., 34, 595 (the ninhydrin reaction method).

According to the foregoing methods, a protected peptide resin having a desired amino acid sequence can be obtained. When an aminomethyl resin derivative is used as the insoluble resin, the protected polypeptide can be removed from the resin, for example, by treating the protected peptide resin with ammonia in an appropriate solvent. The resulting protected peptide is then treated with hydrogen fluoride to obtain a polypeptide amide represented by the above formula and freed of all the protecting groups.

When a benzhydrylamine resin, methylbenzhydrylamine resin, aminomethylphenoxymethyl resin or DMBHA resin [Funakoshi, S. [illegible] resin and the protecting groups can simultaneously be removed from the polypeptide by treating the protected peptide resin with hydrogen fluoride, TFMSA (trifluoromethanesulfonic acid) [Yajima, H. et al.; "The Peptides" vol. 5, page 65 (1983),

polypeptides of the invention exhibit significantly higher anti-HIV activity than known high endotoxin affinity polypeptides (e.g., Tachyplesins I, II or III or Polyphemusins I or II) exhibits.

Recent development of delivery systems capable of efficiently introducing DNA into the target cell has made practical human gene therapy for genetic diseases, cancer, AIDS and etc. [Morgan and Anderson, Annu. Rev. Biochem., 62, 191-217 (1993)].

The DNA-transfection systems include the use of polycations, calcium phosphate, liposome fusion, retroviruses, microinjection, electroporation and protoplast fusion. However, all of these methods suffer from one or more problems related to either cellular toxicity, poor reproducibility, inconvenience, or inefficiency of DNA delivery [Flegner et al., Proc. Natl. Acad. Sci. USA, 84, 7413-7417 (1987)].

Recently, highly efficient DNA-transfection procedure using cationic lipid-DNA complex or cyclic amphipathic peptide-DNA complex [Legendre and Szoka, Jr., Proc. Natl. Acad. Sci. USA, 90, 893-897 (1993)] has been reported. The peptides that can form a transfecting complex with DNA include gramicidin S, tyrocidine, polymyxin B, polylysine and melittin all with cationic nature. Among these, the most effective cationic peptide is gramicidin S which is known as an amphipathic cyclic decapeptide antibiotic with β-sheet conformation and can permeabilize and disrupt cell membranes. Both a positive charge and amphipathic character of gramicidin S are thought to be important for the high transfection. Considering these structural characteristics, the polypeptides of this invention can be an alternative candidate of gramicidin S for DNA complex with high transfecting ability because of their strongly cationic and amphipathic nature with β-sheet conformation.

In fact, tachyplesin I, one of the parent molecule of the polypeptides of this invention, can permeabilize membranes and bind to DNA similarly to gramicidin S [Matsuzaki et al.,

In the examples herein, the synthesis of polypeptide (1) is described. Additionally, the results of anti-HIV activity assays for the polypeptides of the invention and known high endotoxin affinity polypeptides are disclosed. Polypeptides of the invention have a significantly higher anti-HIV activity than known high endotoxin affinity polypeptides.

Apparatuses and reagents used in the following examples are as follows:

HPLC apparatus:

Shimadzu Corporation, Model LC-6AD

Column of the apparatus: Asahipak ODP-90 (Asahi Chemical Industry Co., Ltd.)

Fmoc amino acid and amino resin: produced by Watanabe Chemical Industries, Ltd.

Condensing agent: produced by Peptide Institute, INC. and Applied Biosystems Japan

FAB-MS (FAB-mass spectrograph): VC Co. (USA), Model ZAB-SE

6.1. EXAMPLE 1 : SYNTHESIS OF THE POLYPEPTIDE (1)

The synthesis of a polypeptide (1) which has the formula shown below is described in Sections 6.1.1-6.1.7, infra. Polypeptides (2-13,22,23) and precursor peptides of polypeptides(14-21,24, 25)(see Table I, supra for structures) are synthesized using similar procedures.

1 2 3 4 5 6 7 8 9 10 11 12 13

Arg-Arg-Trp-Cys-Tyr-Arg-Lys -DLys-Pro- Tyr-Arg-Lys-Cys-Arg-NH_2

.....(1)

In the above formula (1), Arg, Trp, Cys, Tyr, Lys, DLys and Pro denote the aforementioned amino acid residue, and the solid line between the Cys at the 3- and 11- positions denotes disulfide linkage.

Likewise as above, Lys(Boc), Arg(Pmc), Tyr(tBu), Pro, DLys(Boc), Lys(Boc), Arg(Pmc), Tyr(tBu), Cys(Trt), Trp, Arg(Pmc) and Arg(Pmc) were successively introduced into the DMBHA resin to obtain a protecting group-protected peptide (1) resin.

Each amino acid condensation reaction in the solid phase synthesis was carried out according to the operation conditions of the Table 2.

TABLE 2

Operation	Reagent	Solvent	Time x Repeat number
Removal of Fmoc Group	20% piperidine/DMF	DMF	5 min. x3
Washing	----	DMF	1 min. x6
Condensation reaction	Fmoc amino acid(2.5 eq) + DIPCDI + HOBt	DMF	2 hr. x1
Washing	----	DMF	1 min. x4

6.1.5. PREPARATION OF THE POLYPEPTIDE (1) BY THE REMOVAL OF THE PROTECTING GROUPS, REMOVAL OF POLYPEPTIDE (1) FROM THE RESIN AND PARTIAL PURIFICATION

The protecting group-protected polypeptide (1) resin was subjected to 20% piperidine/DMF treatment to remove the Fmoc group, and then subjected to reaction at 25°C for 2 hours in a 1 M TMSOTf-thioanisole/TFA(trifluoroacetic acid) system (10 ml of trifluoroacetic acid in the presence of m-cresol (100 eq) and ethanedithiol (300 eq)) per 100 mg of the resin. The resin was filtered off from the reaction mixture and washed twice with 1 ml of trifluoroacetic acid. 100 ml of ice-cooled dry ether was subsequently added to mixture of the filtrate and the washing. The formed precipitate was centrifuged, and the residue was separated from the supernatant by decantation. The resulting residue was washed with cold ether, dissolved in 10 ml of 4N AcOH and 830 mg, 80 eq of dithiothreitol was

The specific rotation $[\alpha]^{20}_{D}$ of the obtained polypeptide was -17.2°(C = 0.11, 1 N acetic acid).

6.2 EXAMPLE 3 : SYNTHESIS OF A POLYPEPTIDE (14) [N-α-ACETYLATION OF THE AMINO TERMINAL AMINO ACID RESIDUE OF THE POLYPEPTIDE (1)]

The synthesis of a polypeptide (14) of the following formula 9 is described in Sections 6.2.1-6.2.2:

[Formula 9]

1 2 3 4 5 6 7 8 9 10 11 12 13

Arg-Arg-Trp-Cys-Tyr-Arg-Lys -DLys-Pro- Tyr-Arg-Lys-Cys-Arg-NH_2

.....(14)

In the above formula (14), Ac-Arg, Arg, Trp, Cys, Tyr, Lys, DLys and Pro denote the aforementioned amino acid residues, and the solid line between the Cys at 3- and 11-positions denote a disulfide bond.

6.2.1 ACETYLATION OF THE PARTIALLY PROTECTING GROUP-PROTECTED PEPTIDE (1) RESIN

1.301g (0.25 mmol) of the protecting group-protected peptide (1) resin obtained at the step (4) (Section 6.1.4) of the aforementioned EXAMPLE 1 was taken in a reaction vessel of a manual solid phase synthesis. After removal of the Fmoc group, the N-terminal amino group was acetylated according to the Hudson method [J. Org. Chem., 53, 617, (1988)] to obtain 1.241g of an N-terminal α-amino acid-acetylated protecting group-protected peptide (1) resin (yield of dry weight, 100%). The procedure is summarized in the following Table 3.

The acetylation was carried out by repeating the above [illegible] reaction became ninhydrin positive.

6.2.2 PREPARATION OF THE PEPTIDE (14) BY THE REMOVAL OF THE PROTECTING GROUPS AND THE RESIN OF THE N-TERMINAL AMINO ACID-ACETYLATED PROTECTING GROUP-PROTECTED PEPTIDE (1) RESIN, PARTIAL PURIFICATION AND OXIDATION

The polypeptide (14) was prepared by the same way as described in 6.1.5 and 6.1.6 supra. The specific rotation $[\alpha]^{20}_{D}$ of the polypeptide (14) was -18.3°(c=0.08, 1N acetic acid). The polypeptide (14) was acid-hydrolyzed in 4M methanesulfonic acid containing 0.2% tryptamine at 115°C for 24 hours according to the method of Liu et al. [J. Biol. Chem., 251, 1936 (1976)]. The result of the amino acid analysis accorded well with the calculated value.

6.3 EXAMPLE 4 : SYNTHESIS OF A POLYPEPTIDE (19) [N-α-FLUORESCEINTHIOCARBAMOYL POLYPEPTIDE (1)]

The synthesis of a polypeptide (19) of the following formula 10 was carried out by N-α-fluoresceinthiocarbamoylation of the amino terminal amino acid residue of the polypeptide (1).

[Formula 10]

1 2 3 4 5 6 7 8 9 10 11 12 13

FTC-Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2

....(19)

In the above formula (19), FTC-Arg, Arg, Trp, Cys, Tyr, Lys, DLys and Pro denote the aforementioned amino acid residues, and the solid line between the Cys at 3- and 11- positions

The antiviral activity against HIV of the polypeptide (1) synthesized in Example 1 was tested and evaluated according to the following method.

HIV-infected MT-4 cells (2.5 x 104 cells/well, multiplicity of infection (MOI) : 0.001) immediately after infection were added together with the test substance with various changes of the concentration to a 96-well microtitre plate. After incubation at 37° C for 5 days in a CO_2 incubator, the number of survivor cells was measured by the MTT method [Pauwels et al.; J. Virol. Methods, 20, 309-321 (1988)]. The antiviral activity is expressed as a concentration at which cell death due to HIV infection is 50% inhibited (EC_{50} : 50% effective concentration). On the other hand, in order to know the cytotoxicity of the test substance on the MT-4 cells, virus-non-infected cells were incubated, likewise as above, together with the test compound with various changes of the concentration. The cytotoxicity is expressed as 50% cytotoxic concentration (CC_{50}) due to the test substance. Further, the rough ratio of CC_{50} to EC_{50}, (CC_{50}/EC_{50}) is expressed as an effective ratio (SI).

The following formula 8 represents the peptide antiviral agent used in comparison with the polypeptide (1):

Formula 8

(Arg-Arg-Trp-Cys-Tyr-Arg-Lys-Cys-Tyr-Lys-Gly-Tyr-Cys-Tyr-Arg-Lys-Cys-Arg-NH_2 ..... T-22)

Table 4 shows the EC_{50}, CC_{50}, and SI values of polypeptide (1), the above peptide(T-22) and anti-HIV agent AZT.

TABLE 4

Test compound		CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Example 2	Polypeptide(1)	49.8	0.0034	14,647
Comparative Example 1	T-22	54.1	0.0099	5,465
Comparative Example 2	AZT (azidothymidine)μM	6.68	0.0001	66,800

TABLE 5

Compound(Mame) & Structure	Physical Property $[\alpha]_D^{19-22}$ (Conc., Solvent)	Anti-HIV Activity CC_{50}(μg/ml)	EC_{50}(μg/ml)	SI(CC_{50}/EC_{10})
(1) Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-17.2 (0.11, 1NAcOH)	65.37	0.0195	3352
(3) Arg-Arg-Trp-Cys-Tyr-Arg-Lys- Lys-Gly-Tyr-Arg-Lys-Cys-Arg-NH_2	- 0.9 (0.04, ")	198.18	0.057	3477
(5) Arg-Arg-Trp-Cys-Tyr-Arg-Lys-Pro-DLys-Tyr-Arg-Lys-Cys-Arg-NH_2	-12.1 (0.06, ")	101.20	0.076	1321
(6) Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DOrn-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-12.5 (0.10, ")	55.93	0.021	2728
(14) Ac -Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-18.3 (0.08, ")	78.14	0.024	3256
(17) Myr-Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-19.1 (0.08, ")	106.60	0.055	1838
(19) FTC-Arg-Arg-Trp-Cys-Tyr-Arg-Lys-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	- 5.9 (0.06, H2O)	38.47	0.001	39285
(22) Arg-Arg-Trp-Cys-Tyr-Arg-Lys(ε-N-Ac)-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-13.6 (0.07, 1NAcOH)	44.26	0.030	1475
(23) Arg-Arg-Trp-Cys-Tyr-Arg-Lys(ε-N-But)-DLys-Pro-Tyr-Arg-Lys-Cys-Arg-NH_2	-12.7 (0.10, ")	138.07	0.0165	11095
Comparative Example :				
T-22		49.4	0.017	2889
AZT		8.53	0.0014	6093

CLAIMS

1. A polypeptide represented by the following formula

1	2	3	4	5	6	7	8	9	10	11	12	13
A_1	- Trp -	Cys	- A_2 -	A_3	- A_3 -	X	- A_2 -	A_3	- A_3 -	Cys	- A_3 -	A_4

or salt thereof in which

A_1 is a basic amino acid residue, or a peptide residue having one or at least two basic amino acids, selected from the group consisting of lysine, arginine and ornithine, said basic amino acid residue or peptide residue in which N-α hydrogen atom of amino terminal end of said amino acid residue may be replaced with an acyl group or a substituted thiocarbamoyl group, forming N-α acyl substituted basic amino acid residue, N-α acyl substituted peptide residue, N-α substituted thiocarbamoyl group substituted basic amino acid residue or N-α substituted thiocarbamoyl group substituted peptide residue;

A_2 is a tyrosine or phenylalanine residue;

A_3 is a lysine or arginine residue;

A_4 is an -OH (derived from a carboxyl group) or an $-NH_2$ (derived from an acid amide group);

X is a peptide residue selected from the group consisting of the peptides represented by D-ornithyl-proline, prolyl-D-ornithine, D-lysyl-proline, prolyl-D-lysine, D-arginyl-proline, prolyl-D-arginine, glycyl-ornithine, ornithyl-glycine, glycyl-lysine, lysyl-glycine, glycyl-arginine and arginyl-glycine, in which the hydrogen atom of the ω-amino group of D-lysine, L-lysine, D-ornithine and L-ornithine may be replaced by an ω-amino acyl group, and said peptide residue is connected to the amino acid residues at the 6th and the 8th positions via peptide bond per se;

Trp is a tryptophan residue; and

Cys is a cysteine residue.

2. The polypeptide or salt thereof of claim 1 in which A_1 is at least one basic amino acid selected from the group consisting of lysine, arginine and ornithine.

D-arginyl-proline, prolyl-D-arginine, glycyl-ornithine, ornithyl-glycine, glycyl-lysine, lysyl-glycine, glycyl-arginine and arginyl-glycine, in which the hydrogen atom of the ω-amino group of D-lysine, L-lysine, D-ornithine and L-ornithine may be replaced by an ω-amino acyl group, and said peptide residue is connected to the amino acid residues at the 6th and the 8th positions via peptide bond per se;

Trp is a tryptophan residue; and

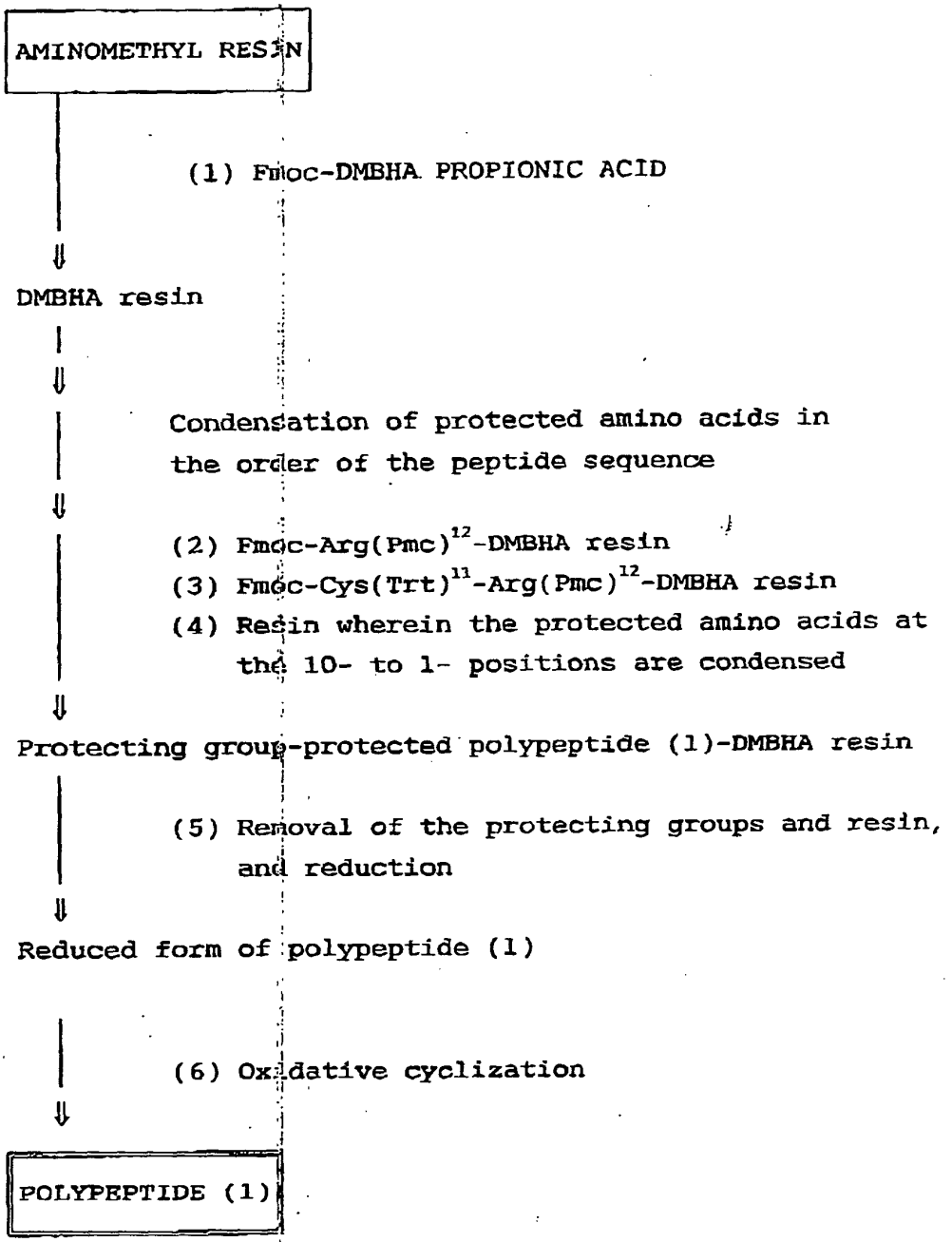
Cys is a cysteine residue;

and the cysteine residues at the 3- and 11- positions are linked through a disulfide linkage.

7. A pharmaceutical composition for inhibiting HIV activity in a patient comprising an effective amount of the polypeptide or salt thereof of claim 1 and a pharmaceutical carrier.

8. A pharmaceutical composition for inhibiting HIV activity in a patient comprising an effective amount of the polypeptide or salt thereof of claim 6 and a pharmaceutical carrier.

FIG. 2


AMINOMETHYL RESIN
(1) Fmoc-DMBHA PROPIONIC ACID
DMBHA resin
Condensation of protected amino acids in the order of the peptide sequence
(2) Fmoc-Arg(Pmc)12-DMBHA resin
(3) Fmoc-Cys(Trt)11-Arg(Pmc)12-DMBHA resin
(4) Resin wherein the protected amino acids at the 10- to 1- positions are condensed
Protecting group-protected polypeptide (1)-DMBHA resin
(5) Removal of the protecting groups and resin, and reduction
Reduced form of polypeptide (1)
(6) Oxidative cyclization
POLYPEPTIDE (1)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/JP 94/01706

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